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Mixed Genogroup SRSV Infections Among a Party of Canoeists Exposed to Contaminated Recreational Water

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Samples of faeces collected from a party of canoeists involved in a gastroenteritis outbreak were examined by electron microscopy and RT-PCR for evidence of infection with SRSVs. A broadly reactive primer pair was used to detect SRSVs followed by application of genogroup-specific primers to SRSV-positive specimens. Exposure data were collected by means of a questionnaire. SRSVs were detected in 1/4 specimens examined by EM and 3/4 by RT-PCR. Genogrouping, and sequencing of PCR products revealed two distinct strains: a genogroup I strain, related to the Desert Shield virus, and a genogroup II strain, related to the Lordsdale virus to be associated with the outbreak. Exposure data indicated that capsising and eating food before getting changed were associated with an increased risk of gastroenteritis and was consistent with infection following the consumption of contaminated water. This study confirms the greater sensitivity of RT-PCR for the diagnosis of SRSV infections and its utility, when incorporating genogroup-specific primers, in establishing more complex epidemiological data. ***J. Med. Virol. 52:425–429, 1997.***

KEY WORDS: SRSVs; gastroenteritis; RT-PCR; genogrouping

INTRODUCTION: DIAGNOSING SRSV INFECTIONS

Small round structured viruses (SRSV) are an increasingly recognised cause of acute gastroenteritis [Kapikian and Chanock, 1996; Blacklow and Greenberg 1991]. Outbreaks are most frequently recognised in adults in semiclosed institutions such as hospitals and residential homes and are characterised by a high attack rate in staff and patients due to extensive person-to-person spread. SRSVs are the most commonly recognised cause of foodborne and waterborne viral gastroenteritis [Hedberg and Osterholm, 1993]; however, the number of outbreaks recognised is small and the true importance of environmental contamination in sustaining SRSV infection in the community remains to be established.

The laboratory diagnosis of SRSV infections is, at present, almost exclusively performed by electron microscopy (EM) (Caul and Appleton, 1982), but this procedure is labour intensive and relatively insensitive, requiring 10^6–10^7 particles/gram (ml) of faeces. Several techniques, including ultracentrifugation, precipitation, and immune EM have been used to improve the sensitivity of detection.

Reverse transcription-polymerase chain reaction (RT-PCR) has more recently been introduced and been shown to improve sensitivity of testing and to add additional epidemiological information. Oligonucleotide primers, constructed for highly conserved regions of caliciviruses (feline calicivirus [Carter et al., 1992], rabbit haemorrhagic disease virus [Meyers et al., 1991]) and SRSVs (Norwalk virus [Jiang et al., 1990], Snow Mountain virus and Southampton virus [Lambden et al., 1993]) have been used in RT-PCR assays for detecting SRSVs in stool samples collected from infected individuals [Jiang et al., 1992; Green et al., 1994; Norcott et al., 1994; Wang et al., 1994; Ando et al., 1995] and the increased sensitivity of RT-PCR over EM has been demonstrated [De Leon et al., 1993]. These assays in conjunction with DNA sequencing have allowed the comparison of partial genomic sequences of SRSVs from different geographical areas [Wang et al., 1994; Ando et al., 1995]. Most published RT-PCRs detect only a small proportion of SRSV strains because of the extensive genomic diversity [Norcott et al., 1994; Ando et al., 1995]. Green et al.,

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[1995] have constructed primers that detect >90% of SRSV strains recently circulating in the UK.

In this study, RT-PCR with broadly reactive primers was used to detect and genomically characterise SRSVs associated with an outbreak of gastroenteritis in a party of canoeists. Simultaneous infection with SRSVs of different genogroups was found and likely to be due to contact with contaminated recreational waters.

MATERIALS AND METHODS

Canoeing Party

A party of 13 people from the Cambridge area visited the National Watersports Centre at Holme Pierrepont, Radcliffe on Trent, in October 1994. Eleven of the thirteen people canoed and seven of the canoeists suffered gastroenteritis within 48 hours of their return. All canoeists were asked to supply relevant information concerning the visit and to complete a questionnaire designed to determine their exposure to possible sources of SRSVs. Basic information was provided by all participants and completed questionnaires were returned by six (5 ill, 1 unaffected) of the party.

Faecal Specimens

Faecal specimens were obtained from four of the affected individuals within 72 hours of developing symptoms. A 10% extract of faeces in 199 balanced salt solution (BSS) (Sigma, Dorset) was prepared for examination by electron microscopy and an aliquot of each was stored at −70°C until later testing by SRSV RT-PCR.

Electron Microscopy

All samples were examined directly [Barrish et al., 1994] and after clarification and ultracentrifugation. For the direct examination a small portion of the faecal specimen was mixed with 3% potassium phosphotungstate (PTA)(pH 6.3). A carbon formvar-coated copper grid was floated on 10 μl of the mixture for 1 minute, blotted to remove excess material, and allowed to dry in air.

A 5 ml aliquot of the 10% faecal suspension (in BSS 199) was clarified by low speed centrifugation (5,000 g) for 10 minutes. The supernatants were collected and centrifuged at 150,000 g for 1 hour in an ultracentrifuge. The resulting pellets were resuspended in 3% PTA and placed on carbon formvar-coated copper grids. All EM grids were examined at a magnification of 50,000 X in a JEOL JEM-100 CX electron microscope.

RNA Extraction and RT-PCR

RNA was extracted from 100 μl of the 10% faecal extract (in BSS 199) as described previously [Green et al., 1993] and eluted in 20 μl of RNase-free sterile water containing 20 units ribonuclease inhibitor (RNAsin, Promega, Madison, WI). One microliter of 50 μM random primers (PdN6, Pharmacia Biotech) was added to the extracted RNA, the mixture was overlaid with mineral oil (400-5, Sigma, St. Louis, MO), heated at 70°C for 5 minutes, chilled on ice, and added to 14 μl of reaction mix consisting of 25 mM Tris-HCl pH 8.3, 125 mM KCl, 12.5 mM $MgCl_2$, 1.25 mM each dNTP (Boehringer Mannheim, Mannheim, Germany), and 100 units M-MuLV reverse transcriptase (FPLC-pure, cloned M-MuLV, Life Technologies, Bethesda, MD). Reverse transcription was performed at room temperature for 10 minutes followed by incubation at 37°C for 1 hour, after which the reaction was terminated by incubation at 95°C for 5 minutes followed by chilling on ice. Five microlitres of this cDNA preparation were then added to 45 μl PCR reaction mix (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 350 μM each dNTP, 1 unit Taq polymerase and 20 pMoles of each primer Ni and E3 [Green et al., 1995]. After denaturation at 94°C for 2 minutes, 30 amplification cycles of 95°C for 1 minute, 40°C for 1 minute, and 72°C for 1 minute were performed followed by a final extension of 72°C for 10 minutes.

TABLE I. Nucleic Acid Sequences of Five Digoxigenin-labelled Probes Used for Southern Blotting

Probe	Sequence (5′- > 3′)
Probe 1	TATGTGCCCTGTAGAAGT
Probe 2	TATCACCTGATGTTATACAATCC
Probe 3	GTCCCCTGACATCATACAGGCT
Probe 4	ATCCCCTGACATCGTCCAGGCT
Probe 5	CTGTCGGAGGTCACGGGGCTTG

Amplification products were examined by electrophoresis of 20 μl reaction mix in agarose gels (4% Nusieve 3:1, Flowgen) at 10 v/cm for 1.5–2 hours. cDNA from samples found negative with the primer pair Ni/E3 was retested in a reaction mix containing primers GI/GII/E3 [Green et al., in prep.]. This combination comprises three primers, two sense primers (GI and GII) that specifically amplify genogroup I and genogroup II strains, respectively, when used in combination with the antisense primer E3 [Green et al., 1995]. Amplicons for genogroup I and II strains are 190bp and 270bp in size, respectively, thus allowing detection and differentiation of genogroup I and II strains in a single assay.

Southern Blot Hybridisation of Amplification Products

Agarose gels were prepared for Southern blot procedure by submersion for 30 minutes in denaturation solution (0.5 M NaOH/1.5 M NaCl) followed by neutralisation (3 M NaCl/0.5 M Tris pH 7.0) for 30 minutes. Amplification products were transferred onto a positively charged nylon membrane (Boehringer Mannheim) using a standard capillary procedure and cross-linked to the membrane by baking at 120°C for 20 minutes. After prehybridisation for 1 hour at 40°C (5 × SSC, 0.1% sarkosyl, 0.02% SDS, 1% Boehringer Mannheim blocking reagent), a mixture of five digoxigenin-labelled probes (Table I) derived from genomic sequences of SRSV strains detected in the UK were added to the hybridisation mix and hybridisation occurred overnight at 40°C. After two 5-minute washes in

TABLE II. Exposure Data and Clinical Details Obtained From Canoeists

Canoeist	Age	Canoeing	Time on river (hours)	Submerged	Ill	Onset date	Onset time	Duration	Symptoms[a]
CH	32	Yes	4	Yes	Yes	23.10.94	23.30	36	D, V, C
JC	40	Yes	4	Yes	Yes	24.10.94	1.45	24	D, V, C
ST	16	Yes	2.5	Yes	Yes	23.10.94	10.00	36	D, V, C
AD	25	Yes	3	Yes	Yes	23.10.94	22.00	8	D, V, C
RT	14	Yes	4	Yes	Yes	24.10.94	5.00	Not stated	D, P
MD	15	Yes	4	Yes	Yes	23.10.94	20.30	100[b]	D, V
LW	16	Yes	3	Yes	Yes	23.10.94	7.30	12	V
WR	18	Yes	4	Yes	No	NA	NA	NA	NA
RH	28	Yes	4	Yes	No	NA	NA	NA	NA
DB	23	Yes	3	Yes	No	NA	NA	NA	NA
RHu	26	Yes	4	No	No	NA	NA	NA	NA
LH	28	No	NA	NA	No	NA	NA	NA	NA
AC	13	No	NA	NA	No	NA	NA	NA	NA

[a]D: diarrhoea; V: vomiting; C: cramps; P: pyrexia; NA: not applicable.
[b]Diabetic.

2 × SSC, 0.1% SDS, hybridised probe was detected by chemiluminescence. Hybridisation and chemiluminescence detection reagents were as manufacturers' recommendations (Boehringer Mannheim).

Sequencing of PCR Products

Unincorporated deoxynucleotides and primers were separated from amplification products on a chromaspin 100 column (Cambridge Bioscience) according to manufacturer's instructions. The DNA sequence was determined using an ABI 373A automated sequencer and a Taq FS Cycle sequencing kit (Applied Biosystems, Foster City, CA). The amplicons were sequenced in both directions with either the GI and E3 or GII and E3 primers, according to the genogroup of the strain detected.

RESULTS

Data obtained immediately after the outbreak indicated that seven of the party suffered gastroenteritis within 48 hours of their return. Four of the seven (57.1%) experienced diarrhoea, vomiting, and stomach cramps, one (14.3%) had diarrhoea and vomiting, one had diarrhoea and pyrexia, and one had vomiting only (Table II). The median duration of illness was 36 hours (range 8–100 hours). A diabetic individual was unwell for 100 hours. All affected individuals were on the water during the trip, and the party did not stay overnight at the centre nor eat food prepared there.

Exposure data from six people (5 ill and 1 not ill) who used the course was obtained by a questionnaire. Two of the party did not canoe and only walked along the bank. One of these (AC) shared the same meal as one of the affected individuals (JC) but remained well. All five people who reported swallowing water became ill compared with none of those who did not get ill ($P < 0.02$ Fisher exact test). The same five people also ate food by the course before getting changed ($P < 0.02$). The meal was in the form of packed lunches prepared by the individuals before leaving home and with no common constituents.

TABLE III. Electron Microscopy and SRSV RT-PCR Results

Canoeist	EM	RT-PCR Ni/E3	RT-PCR GI/GII/E3 genogroup
JC	Neg	Neg	Neg
ST	Neg	Pos	GII
CH	Pos	Pos	GI
AD	Neg	Pos	GII

Electron Microscopy

SRSVs were seen by direct examination and ultracentrifugation in one faecal specimen (Table III).

Microbiological Indicators of Water Quality

Routine sampling of the River Trent on the canoe slalom course, performed on 25 October, 2 days after the visit, showed 1,020 *Escherichia coli* and 212 enterococci per 100 ml, and the numbers of these organisms continued to increase to 23,800 *E. coli* and 3,440 enterococci per 100 ml up to 14 November. Water samples were not collected for virological examination at that time.

RT-PCR for SRSV

Three of the four faecal specimens were RT-PCR positive using the Ni/E3 primer pair and were confirmed by Southern blot hybridisation. RT-PCR performed with the genogroup specific oligonucleotide primers GI/GII/E3 indicated that two of the three SRSV RNA-positive samples contained genogroup II strains and the third a genogroup I strain (Table III).

Sequencing of PCR Products

A 156bp region of the RNA polymerase gene (relating to the intraprimer region of GI/E3) from both the genogroup I and genogroup II strains was analysed. The genogroup I strain was found to be most closely related the Desert Shield virus [Lew et al., 1994] with 75% identity at the nucleotide level. The genogroup II strain

Fig. 1. Dendogram of predicted genetic relationships of the canoeing outbreak viruses among different SRSVs by comparison of a part of the polymerase region within ORF1.

was most closely related to Lordsdale virus (Dingle et al., 1995) with 92% identity in this region (Fig. 1).

Alignment of the nucleic acid sequences of RT-PCR GI/E3 and GII/E3 amplicons obtained from ST and CH (Table III), with published sequences over a 164 base region are shown in Figure 2.

DISCUSSION

The facilities at Holme Pierrepont include a 2 km rowing course, a lake used for water skiing, and a white water canoe slalom course. These waters are supplied from a variety of sources including surface streams and the River Trent. There have been a number of reports of illness among groups of users at the National Water Sports Centre; the reported illness in users usually lacked microbiological confirmation. Very few specimens were sent for microbiological investigation, and no specimens for virological examination have been received at the local Public Health Laboratory from users of the course who have developed illness. Many people consulted their GP only after the time viruses could be found in the faeces by routine methods. Linking these illnesses to the water with certainty has been difficult as only a small number of cases of illness are reported among the large number of users. When a number of people in a group develop illness, they have invariably had social contact or eaten the same food; therefore, other possible explanations for the clusters of illness cannot be easily eliminated. In this group of canoeists, however, the illness was confirmed to be due to infection with SRSVs and affected those who ingested contaminated water from the River Trent, which supplies the slalom course.

In the group, there were individuals who attended the centre but did not canoe and who did not report illness and thus acted as internal controls for social

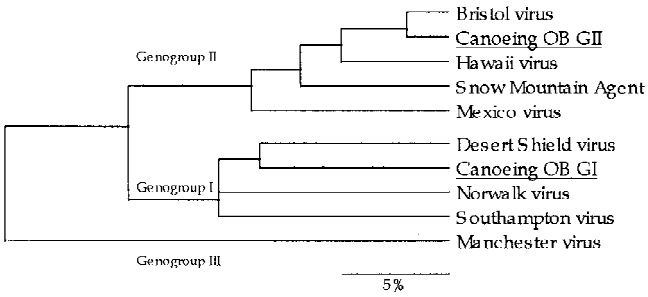

Fig. 2. Alignment of the nucleic acid sequences of RT-PCR GI/E3 and GII/E3 amplicons obtained from ST and CH (Table III) with published sequences over a 164 base region corresponding to bases 4688–4851 of the Norwalk virus genome.

factors. Also, two distinct SRSVs, one from each genogroup, were detected in stool samples from the affected members of the group. This is consistent with SRSV infection acquired from sewage-contaminated water as a similar observation has been documented in SRSV infections following the consumption of oysters reared in sewage-contaminated oyster beds [Sugieda et al., 1996].

The presence of two different SRSV genogroups in affected members of the party of canoeists, the simultaneous onset of illness and illness among those ingesting water through submersion while canoeing or eating food before getting changed are strong evidence for the ingestion of contaminated water from the River Trent being the source of the illness and not social factors. Factors such as swallowing water while canoeing and eating before getting changed are associated with increased risk of gastroenteritis among users of Holme Pierrepont [Lee et al., 1996].

This study confirms the greater sensitivity of RT-PCR for the diagnosis of SRSV infections with 75% of samples reactive in the RT-PCR compared with only 25% shown to contain virus by EM. Although water samples were not available during the outbreak described, the improved sensitivity offered by PCR may make it possible to investigate environmental samples, including water, implicated in outbreaks of gastroenteritis.

The combination of initial testing with the broadly reactive primer pair (Ni/E3) followed by genogroup-specific primers (GI/GII/E3) allows detection and genogrouping of SRSVs of wide genomic diversity relatively quickly. This study highlights the potential of RT-PCR in investigations of environmental contamination and will provide an essential tool in determining the impact of environmental contamination on the epidemiology of SRSV infection in the community.

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